

**Antidiabetic actions of aqueous bark extract of *Swertia chirayita* on insulin secretion, cellular glucose uptake and protein glycation**

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**Running title:** Antidiabetic actions of *Swertia chirayita*

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## ABSTRACT

**Objective:** There is renewed scientific interest in the potential of plant-derived agents for the treatment of diabetes mellitus. This study investigated the antidiabetic actions of *Swertia chirayita*, a plant used traditionally in the management of diabetes.

**Methods:** Insulin secretion from BRIN-DB11 cells was assessed in the absence or presence of plant extract and modulators of beta cell function. Glucose uptake was assessed using 3T3-L1 cells while effects of the plant extract on protein glycation was assessed using model peptide. Insulin was measured by radioimmunoassay and intracellular calcium by FLEXstation<sup>TM</sup>.

**Results:** *Swertia chirayita* stimulated concentration-dependent insulin secretion from BRIN-BD11 cells ( $P < 0.001$ ). Its insulinotropic effects were abolished in the absence of extracellular  $\text{Ca}^{2+}$  or by diazoxide ( $P < 0.001$ ,  $n=8$ ) and were significantly decreased by verapamil and in beta cell depolarization by with KCl. *Swertia chirayita* extracts evoked a 28 – 59% increase in basal and insulin-stimulated glucose uptake by 3T3-L1 cells. Protein glycation was significantly ( $P < 0.05$  –  $P < 0.001$ ) inhibited by *S. chirayita* in a dose-dependent manner.

**Conclusion:** This study reveals that the antidiabetic actions of *S. chirayita* aqueous bark extracts involves the stimulation of insulin secretion and enhancement of insulin action. Inhibition of protein glycation may also help counter diabetic complications. These actions of *S. chirayita* may provide new opportunities for the treatment of diabetes.

## Introduction

Diabetes mellitus is a major health problem, which is emerging as a pandemic contemporaneous with changes in lifestyle and longer life-expectancy [1]. Treatment of diabetes is advancing on a number of fronts, ranging from better understanding of the mechanisms of action of existing agents to the development of new drugs [2]. Regimens currently employed include lifestyle modification, oral-antidiabetic drugs and more widespread use of insulin therapy. Despite their usefulness, none of these treatments either alone or/in combination can mimic physiological insulin release or prevent the development of chronic complications [3]. Nature is an exemplary source of medications and indigenous remedies have been used in the treatment of diabetes mellitus in many parts of the world since the 6<sup>th</sup> century BC [4]. Use of traditional medicines in the treatment of diabetes mellitus is probably based primarily on alleviation of its obvious symptoms such as pronounced thirst and polyuria. Recently, there has been a renaissance of clinical, pharmaceutical and scientific interest in the potential of plant treatments. However out of an estimated 250,000 higher plants, it is estimated that less than 1% has been screened pharmacologically [5,6].

*Swertia chirayita* is a medicinal plant indigenous to temperate Himalaya and its medicinal usage has been reviewed previously [7]. It is widely esteemed in Ayurvedic medicine and also used as bitter tonic in treatment of fever and various skin diseases [7]. An increasing body of evidence also support the use of this herb in the treatment of diabetes [8]. Swerchirin (1:8 dihydroxy 3:5 dimethoxy xanthenes), derived from the hexane fraction of *Swertia chirayita*, has demonstrated anti-hyperglycaemic activity in fasted, fed, glucose-loaded, tolbutamide pre-treated and STZ-induced diabetic rats [9-11]. However, mechanisms by which *Swertia chirayita* ameliorates hyperglycaemia are not fully understood. The present study investigated

the effects of aqueous extracts of *Swertia chirayita* bark on insulin secretion and glucose uptake at the cellular level and assessed its effects on protein glycation *in vitro*.

## **Materials and Methods**

### **Plant material**

Dried bark of *Swertia chirayita* was obtained from a commercial supplier, Cure Herbs Pvt, Delhi, India. A voucher specimen of the plant is available. Bark was homogenised to a fine powder and stored in opaque screw-top jars at room temperature ( $20\text{ }^{\circ}\text{C} \pm 2\text{ }^{\circ}\text{C}$ ) until use. For *in vitro* studies, an aqueous decoction was prepared by bringing 25 g/L of material to the boil in distilled water. Once boiling, the suspension was removed from the heat and allowed to infuse over 15 minutes. The suspension was filtered (Whatman no.1 filter paper) and the volume adjusted so the final concentration was 25 g/L. 1 mL aliquots of the filtered plant solution were brought to dryness under vacuum (Savant Speedvac, Savant Instrumentation Incorp., NY, USA). Dried fractions were stored at  $-20\text{ }^{\circ}\text{C}$  until required. Fractions were reconstituted in incubation buffer for subsequent experiments as required.

### **Insulin secretion**

Insulin release was determined using monolayers of BRIN BD11 clonal pancreatic cells [12]. BRIN-BD11 cells were grown in RPMI-1640 tissue culture medium containing 11.1 mmol glucose/L, 10% fetal calf serum and antibiotics (50,000 IU penicillin-streptomycin/L), and maintained at  $37\text{ }^{\circ}\text{C}$  in an atmosphere of 5%  $\text{CO}_2$  and 95% air. Cells were harvested and seeded in 24 - well plates at a density of  $1.0 \times 10^5$  cells per well. Following overnight attachment, culture medium was removed and cells were pre-incubated for 40 min at  $37\text{ }^{\circ}\text{C}$  with 1 mL of Krebs Ringer bicarbonate

(KRB) buffer (115 mM NaCl, 4.7 mM KCl, 1.28 mM CaCl<sub>2</sub>, 1.2 mM KH<sub>2</sub>PO<sub>4</sub>, 1.2 mM MgSO<sub>4</sub>, 10 mM NaHCO<sub>3</sub> and 0.1 % (w/v) bovine serum albumin, pH 7.4) supplemented with 1.1 mM glucose. Subsequent test incubations were performed for 20 min at 5.6 mM glucose using similar buffer containing aqueous plant extract and the agents indicated in Figures. Samples were stored at –20 °C for insulin radioimmunoassay [13]. Cell viability was assessed using a modified neutral red assay as described previously [14].

### **Intracellular calcium ([Ca<sup>2+</sup>]<sub>i</sub>)**

Effects of aqueous extracts of *Swertia chirayita* and other stimuli on intracellular calcium concentration ([Ca<sup>2+</sup>]<sub>i</sub>) in BRIN-BD11 cells were determined fluorometrically as previously described [15-16] using FLIPR [Ca<sup>2+</sup>]<sub>i</sub> (Molecular Devices, Sunnyvale, CA, USA) according to the manufacturer's protocol. Data were captured using a FlexStation scanning fluorometer with integrated fluid transfer workstation (Molecular Devices).

### **Adipocyte differentiation and cellular glucose uptake**

Effects of extracts of *Swertia chirayita* on cellular glucose uptake were assessed using 3T3-L1 fibroblasts (American Type Culture Collection, ATCC, Virginia, USA) as previously described [16, 17]. Briefly, cells were seeded in 12-well plates at a density of 1.0 x 10<sup>5</sup> cells per well, maintained at 37°C ± 2 °C with 5% CO<sub>2</sub> and grown in DMEM culture medium supplemented with penicillin (50 U/mL), streptomycin (50 µg/mL) and fetal bovine serum (10 % v/v). Culture medium was changed every 48 h. Adipocyte differentiation was initiated by the addition of 1 µg/mL insulin, 0.5 mM IBMX and 0.25 µM dexamethosone (Mathews et al., 2006). Differentiated cells were incubated in serum free DMEM for 2 h to establish basal glucose uptake prior to acute

test. Cellular glucose uptake was determined for 15 min at 37°C using KRB buffer supplemented with tritiated 2-deoxyglucose (0.5  $\mu$ Ci/well), glucose (50 mM), insulin and other test agents as indicated in the Figures.

### **Protein glycation**

Effects of aqueous extracts of *Swertia chirayita* on in vitro protein glycation was assessed using insulin as a model substrate as previously described [16, 18]. Human insulin (1 mg/ml, 100  $\mu$ l) was incubated for 24 h in sodium phosphate buffer (10 mM, pH 7.4) containing sodium cyanoborohydride (1.35 mM dissolved in 10mM NaOH) and D-glucose (220 mM) in the presence or absence of graded concentrations of plant extract. The reaction was stopped by addition of acetic acid (0.5 M). Glycated and non-glycated insulin were separated and quantified using reversed-phase high performance liquid chromatography [18].

### **Statistical analysis**

All results are expressed as mean  $\pm$  SEM for a given number of observations (n). Groups of data were compared statistically using one-way ANOVA followed by Newman-Keul post hoc comparison for data sets with  $p < 0.05$ .

## **RESULTS**

### **Insulin secretion studies**

Insulin-release from BRIN BD11 cells, following incubation with aqueous extract of *Swertia chirayita* at 5.6mM glucose, increased significantly in a dose-dependent manner at concentrations  $\geq 0.01$  mg/ml) (Figure 1A) without affecting cell viability (Figure 1B). Insulin secretion was also augmented by *Swertia chirayita* extracts 1.1 mM) and 16.7mM glucose ( $P < 0.001$ ,  $n = 8$ , Figure 2). Stimulation of insulin secretion by *Swertia chirayita* (0.5 mg/ml) were completely abolished in the presence of

diazoxide (300 $\mu$ M) and diminished in the presence of verapamil (50 $\mu$ M, Figure 2). Furthermore, the extract produced a significant 2-fold increase ( $P<0.001$ ) in insulin secretion in the presence of IBMX and its effects were significantly ( $P<0.001$ ) augmented in the presence of 200 $\mu$ M tolbutamide (Figure 2). However, the extract did not significantly increase insulin secretion in cells depolarised with 30 mM KCl (Figure 2) and in the absence of extracellular calcium (Figure 3).

### **Intracellular calcium [ $\text{Ca}^{2+}$ ]<sub>i</sub> studies**

*Swertia chirayita* plant extract (1mg/ml) induced a sharp rise followed by a sustained increase in [ $\text{Ca}^{2+}$ ]<sub>i</sub> (Figure 4A). Addition of verapamil (50 $\mu$ M) resulted in a marked and sustained reduction in the sharp rise of [ $\text{Ca}^{2+}$ ]<sub>i</sub> (Figure 4B).

### **Glucose uptake in vitro**

*Swertia chirayita* (1mg/ml) produced a 28% increase in glucose uptake compared to unsupplemented control ( $p<0.01$ , Figure 5). Glucose uptake increased by 59% when insulin was combined with the plant extract compared with effects observed in the presence of insulin alone (Figure 5).

### **Protein glycation in vitro**

Aqueous extract of *Swertia chirayita* produced a dose-dependent inhibition of insulin glycation. The lowest inhibition of 8% was observed at the extract concentration of 1mg/ml while a maximum inhibition of 25% was observed in the presence of 50mg/ml plant extract ( $P<0.001$ , Figure 6).

## Discussion

Scientific studies providing evidence for therapeutic efficacies of many plant materials used in ethno-medicinal practices are increasing [16, 19]. In the case of *Swertia chirayita*, pharmacological actions such as antipyretic, analgesic, anthelminthic and antiperiodic effects have been reported [20]. Studies reporting hypoglycaemic and antidiabetic effects of various extracts of the plant are also increasing. For instance, Kar *et al* [19] reported that ethanolic extracts of *Swertia chirayita* significantly lowered blood glucose in rats with alloxan-induced diabetic rats. In addition, several studies have implicated swerchirin (3,5-Dimethoxy-1,8-dihydroxyxanthone) isolated from the plant as responsible for its hypoglycaemic effects [21-23]. In agreement with these reports, our present study showed that aqueous extract of *Swertia chirayita*, stimulated insulin secretion in a concentration-dependent manner from BRIN BD11 cells without affecting cell viability.

Though reports of the actual mechanism of hypoglycaemic actions of extracts of *Swertia chirayita* or swerchirin are generally absent, the observed lack of cellular cytotoxicity observed in the present study indicates that the insulintropic effect of the plant extract was not due to simple leakage of insulin from the cells. Moreover, inhibition of the insulintropic activity in the absence of extracellular calcium or with diazoxide strongly suggests the involvement  $\text{Ca}^{2+}$  influx and the  $\text{K}_{\text{ATP}}$ -dependent pathway in the stimulatory actions of the plant extract [16, 24-25]. Indeed, exposure of BRIN-BD11 cells to *Swertia chirayita* produced a substantial rise of intracellular calcium concentration  $[\text{Ca}^{2+}]_i$  which was attenuated but not completely abolished by the calcium channel blocker, verapamil. The ability of extract to enhance the insulintropic actions of tolbutamide further supports involvement of the voltage dependent calcium channels (VDCC) and  $\text{K}_{\text{ATP}}$ -channels. This ability of



sulphonylureas to augment the action of *Swertia chirayita* has been reported in a number of other studies [9, 26].

In addition to its prominent beta cell stimulatory effects, aqueous extract of *Swertia chirayita* enhanced basal cellular glucose transport in differentiated 3T3-L1 adipocytes. The stimulatory action of insulin ( $10^{-9}$  M) was also augmented in the presence of 1mg/ml of extract. The combined actions of the extract and insulin were greater than the effects of either agent alone. Such observations are consistent with previous studies by Saxena *et al.* [10] which demonstrated increased glucose uptake and glycogen synthesis in diaphragm muscle in rats administered with *S. chirayita* extracts. Furthermore, the additive effect of *Swertia chirayita* on glucose uptake may suggest actions outside the normal insulin-signalling pathway. Further studies are needed to assess the possible novelty of the mechanisms involved.

In the final series of experiments, *Swertia chirayita* extract was shown to inhibit protein glycation using insulin as a model substrate [18]. Interestingly, aqueous and ethanolic extracts of *Swertia chirayita* have been reported to contain a number of potent antioxidants such as xanthenes, glycosides, triterpenoids [29] and magniferin [30], which acts at different levels of the oxidation sequence [31]. Thus, the antiglycation actions apparent in the present investigation may be due to its high antioxidant content. This finding also presupposes that treatment with *S. chirayita* may be beneficial in preventing the onset diabetic complications.

In conclusion, the current investigation corroborates earlier reports of the antidiabetic actions *Swertia chirayita* [9-11]. In addition, this study has shown that extract of aqueous bark extract of *Swertia chirayita* stimulated both the secretion and action of insulin as well as inhibited protein glycation. The ability of plant constituents to

influence these parameters in *vivo* depends entirely on soluble active principle(s) being absorbed via the gut and will form the focus of our future studies.

### **Acknowledgement**

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### **Conflict of Interest Statement**

The authors declare that there is no conflict of interest

### **Authors' Contributions**

HT made significant contribution to acquisition of data and analysis. OOO contributed to data analysis and drafting of the manuscript. PRF and YHA designed the experiments and contributed to interpretation of data, drafting and revising of the manuscript for intellectual content. All authors read and approved the final manuscript.

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## Legends to Figures:

Figure 1: Dose-dependent effects of *Swertia chirayita* (A) on insulin secretion and beta cell viability (B) in BRIN BD11 cells. Values are mean  $\pm$  SEM of 8 separate observations. \*\*\*P<0.001 compared to the control.

Figure 2: Modulation of *Swertia chirayita* extract-induced insulin secretion by established stimulators and inhibitors of beta cell function. Values are mean  $\pm$  SEM of 8 separate observations. \*\*P<0.01, \*\*\*P<0.001 compared to compared to 5.6 mM glucose. +++P<0.001 compared to 16.7mM glucose.  $\Delta\Delta$ P<0.01,  $\Delta\Delta\Delta$ P<0.001 compared to the respective incubations in absence of plant extract.

Figure 3: Effects of extracellular calcium on insulin release stimulated by *Swertia chirayita*. Values are mean  $\pm$  SEM of 8 separate observations. \*\*\*P<0.001 compared to 5.6mM glucose in the presence of  $\text{Ca}^{2+}$ .  $\Delta\Delta\Delta$ P<0.001 compared to the respective concentration in the presence of  $\text{Ca}^{2+}$ . +++P<0.001 compared to 5.6mM glucose in the absence of  $\text{Ca}^{2+}$ .

Figure 4. Effects of *Swertia chirayita* on intracellular  $\text{Ca}^{2+}$  in the absence (A) and presence of verapamil (B). Values are mean  $\pm$  SEM of 6 separate observations.

Figure 5: Effects of *Swertia chirayita* extracts on 2-deoxy-D-[ $\text{H}^3$ ]-glucose transport. Results are mean  $\pm$  SEM of 4 separate observations. \*\*P<0.01, \*\*\*P<0.001 compared with incubations in the absence of insulin. +++P<0.001 compared to  $10^{-9}$  M insulin alone.  $\Delta\Delta$ P<0.001 compared to *Swertia chirayita* incubations without insulin.

Figure 6: Effects of *Swertia chirayita* extract on protein glycation. Results are mean  $\pm$  SEM of 3 separate observations. \*\*P<0.01, \*\*\*P<0.001 compared to glycation in the absence of plant extract.

Figure 1

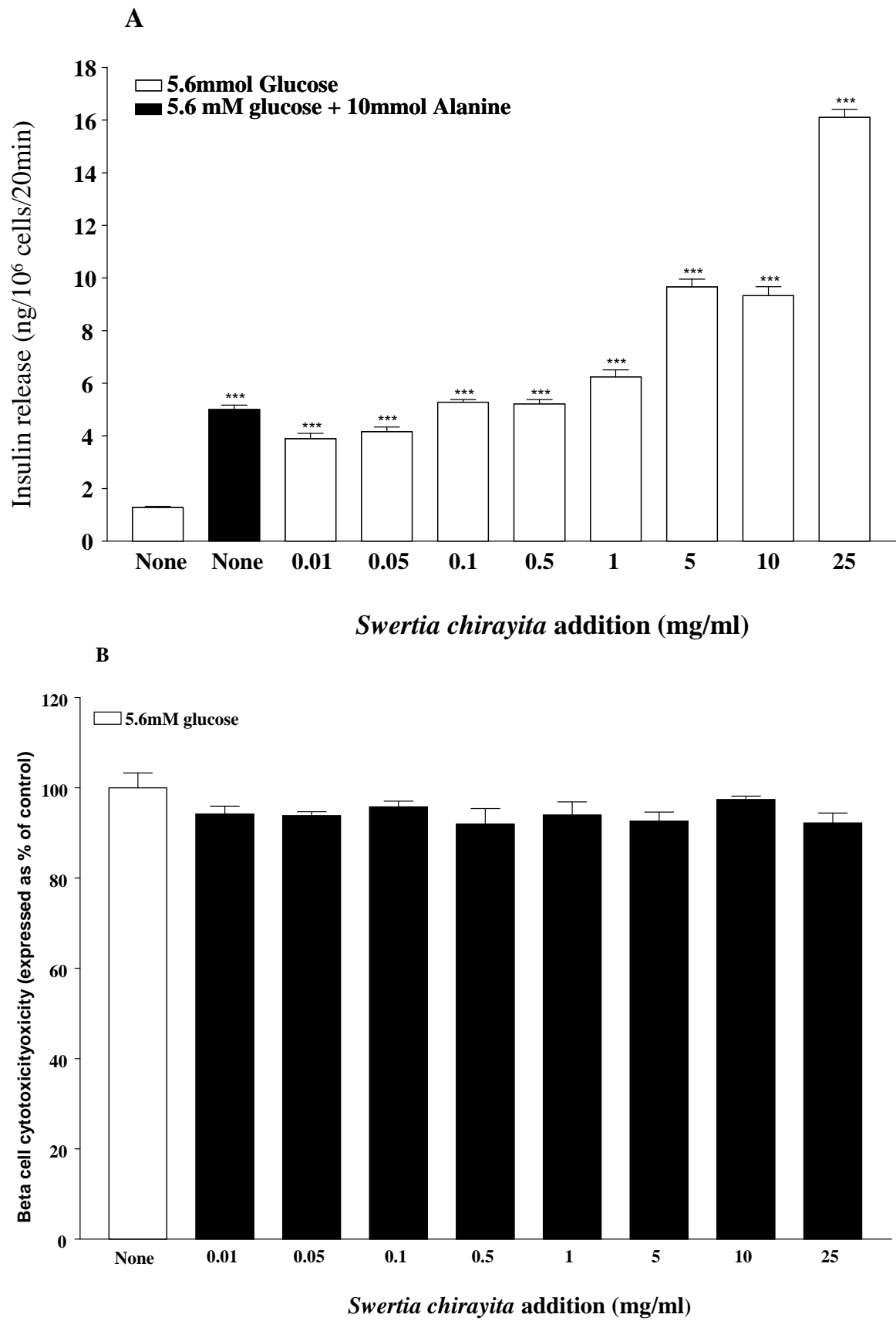


Figure 2

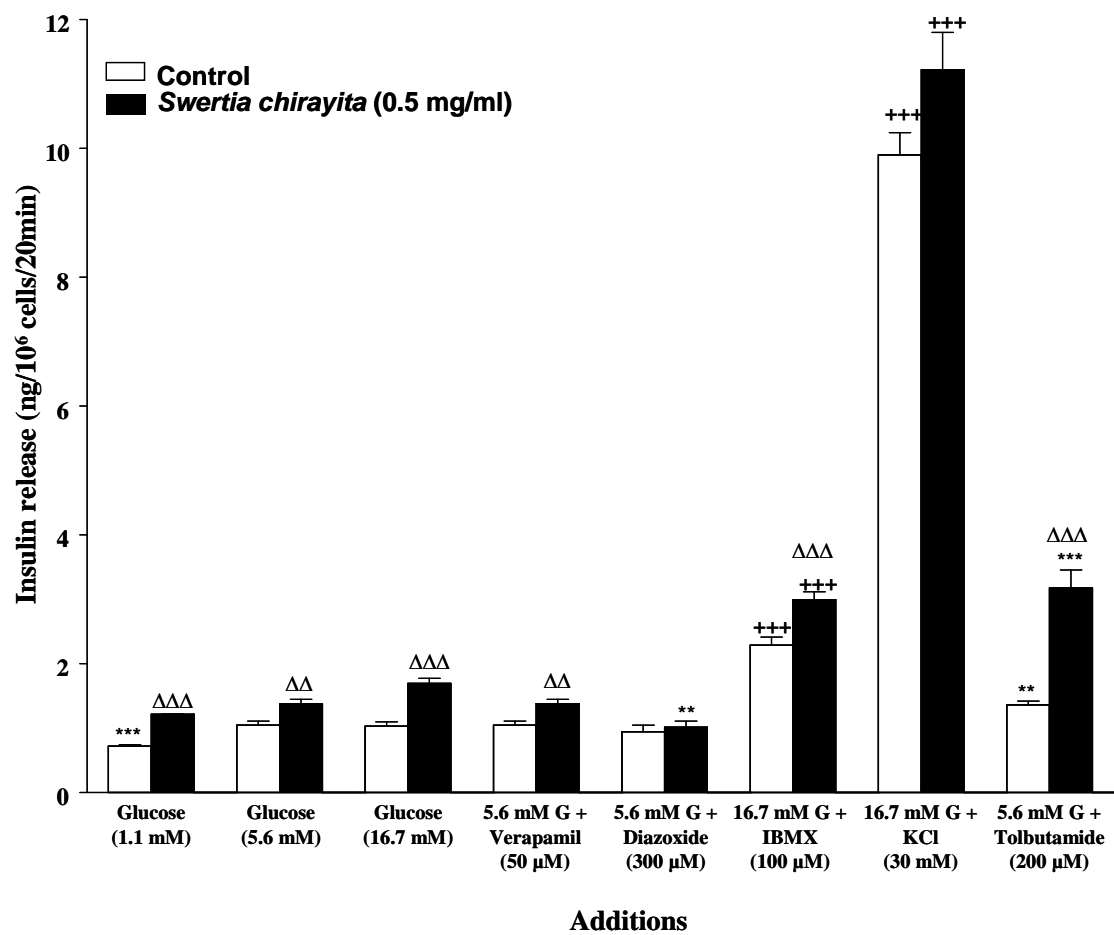




Figure 3

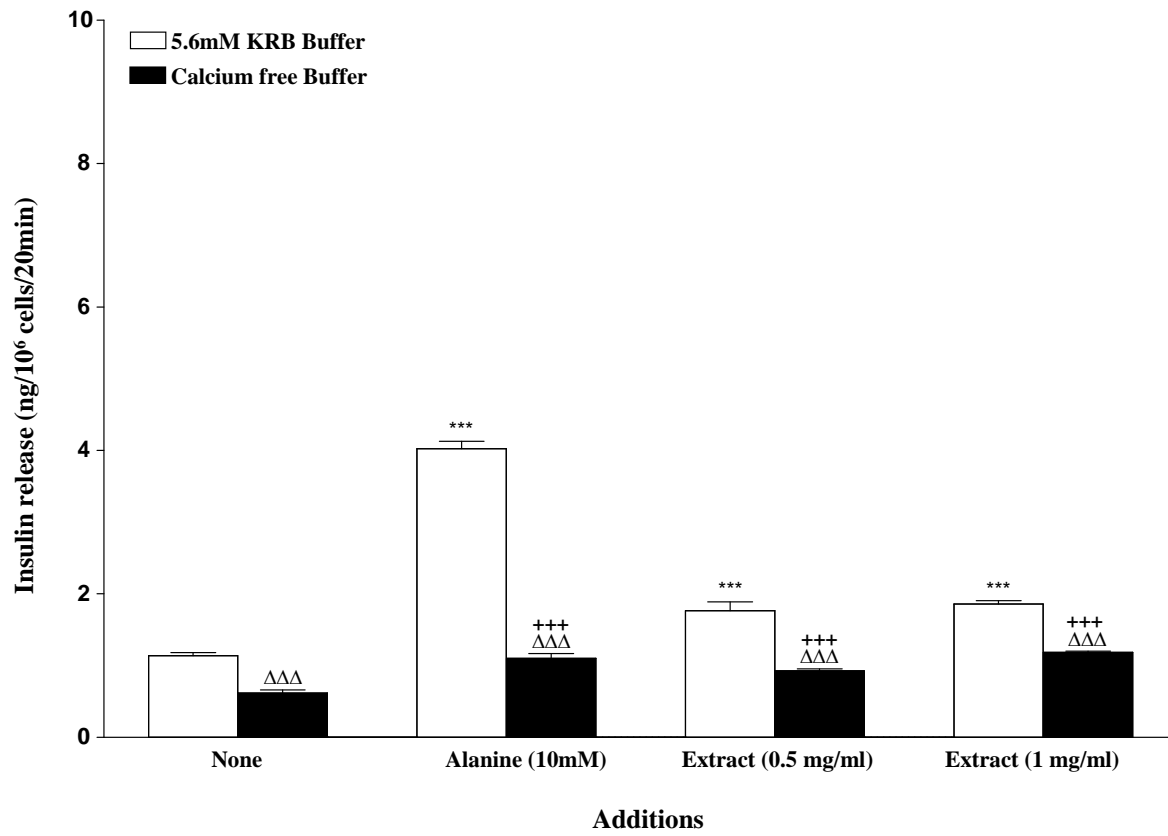


Figure 4

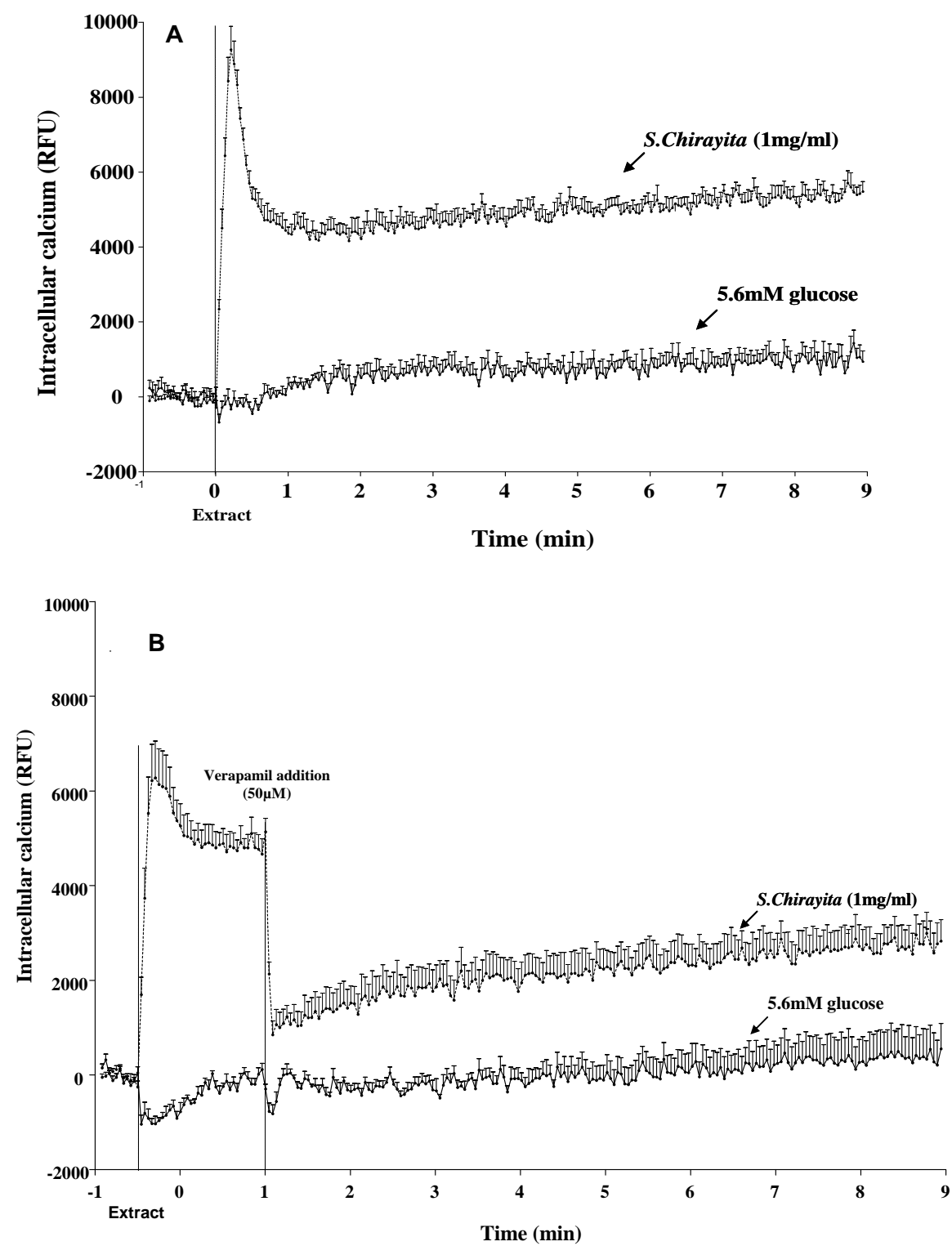


Figure 5

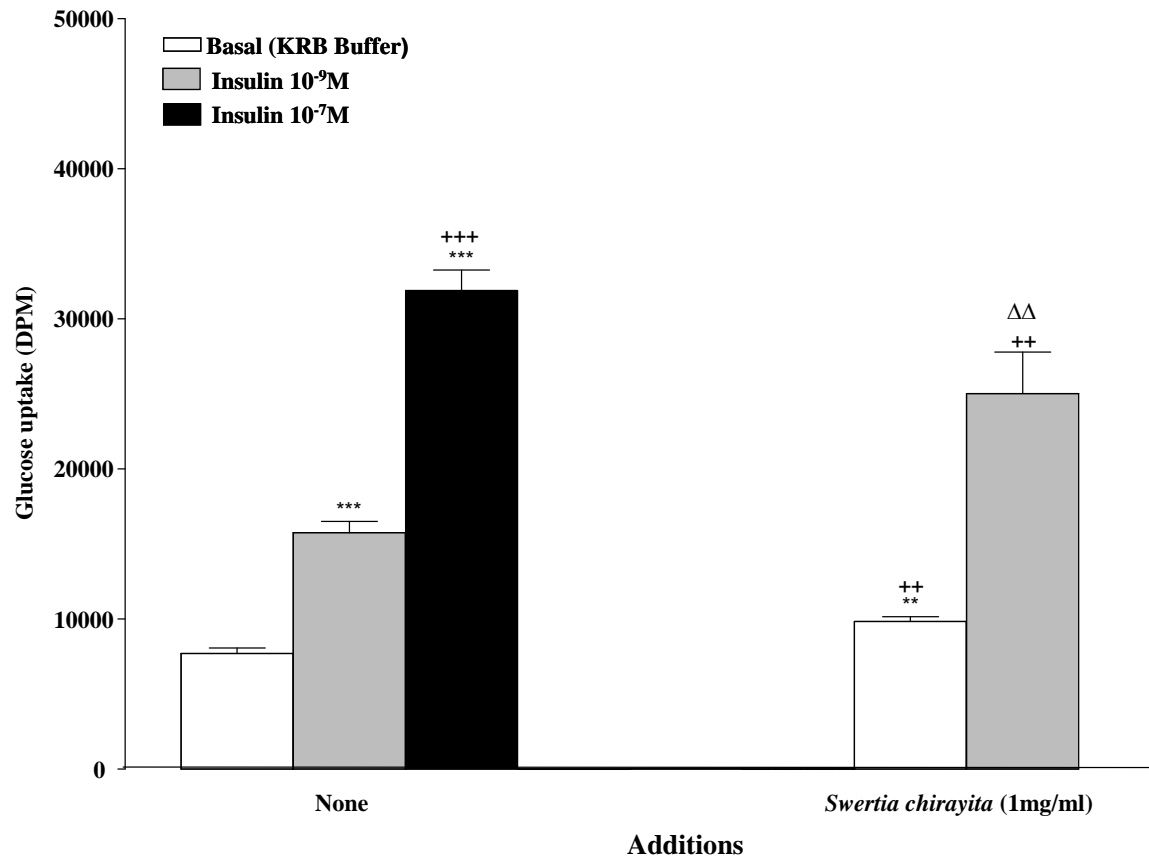


Figure 6

